A study of the possible role of taurine, a cystine catabolic product, on adipogenesis in vitro

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Taurine and adipogenesis

Abstract
Aim: Taurine is a sulfur-containing product of the sulfur amino acid cysteine via the inducible enzyme cysteine dioxygenase (CDO). Plasma cysteine is associated with human obesity, but it is unknown whether this is mediated by cysteine itself or also through its catabolic products.
The aim of this study was to test the role of taurine in adipogenesis through inducing adipogenic differentiation of stromal vascular fraction (SVF) progenitors and preadipocytes in cystine-limiting media with and without taurine supplementation.
Material and Methods: Adipogenic differentiation was induced at limiting cystine concentrations with or without 30 μM taurine supplementation. Oil Red O staining of accumulated lipid and mRNA expression of PPARG1 were assessed as endpoints of adipogenic differentiation. mRNA expression of CDO1 was also measured.
Results: From day 3 of differentiation, small lipid droplets started to appear. On day 8 of differentiation, the cells rounded up to a more spherical shape, and clusters of mature adipocytes filled with lipid droplets were clearly visible. Oil Red O staining showed no difference in lipid accumulation in cells differentiated with or without taurine supplementation (percent area stained = 4.6% vs. 4.7%, P= 0.97), and no difference in lipid droplet size (P= 0.25). Gene expression analysis also revealed no significant effect of taurine supplementation on mRNA expression of PPARG1 (P= 0.92) or CDO1 (P= 0.086).
Discussion: In conclusion, taurine supplementation had no significant influence on adipogenesis in the presence of limiting cysteine concentrations. The mechanistic effects of different sulfur-containing compounds on human adipogenesis deserve further investigations.

Keywords
Cystine; Taurine; Adipogenesis; Obesity; Sulfur Amino Acids

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Introduction
Sulfur amino acids (SAA) play a crucial role in the maintenance of cellular redox state and capacity to detoxify free radicals, reactive oxygen species and other toxic compounds [1]. Methionine and cysteine are the two primary sulfur amino acids in mammals. Methionine is an essential amino acid, obtained from the diet, while cysteine is a semi-essential amino acid synthesized from methionine [1]. Taurine is the most abundant free amino acid in the body and is synthesized in mammals in 2 ways: either from the oxidation of cysteine via cysteine dioxygenase (CDO), or from the oxidation of cysteamine by cysteamine (2-aminoethanethiol) dioxygenase (ADO) [2].

Emerging evidence suggests that SAA are linked to energy metabolism in humans [3]. Plasma total cysteine was correlated with obesity and fat mass in humans [3], dietary animal models also suggest a role of cysteine in the regulation of body fat mass [4]. In vitro, ascending cystine concentrations enhanced adipogenic differentiation of 3T3-L1 cells in a dose-dependent manner [5]. Cysteine dioxygenase (CDO) catabolizes excess cysteine and is necessary for hypotaurine/taurine production from cysteine. A number of animal studies have revealed anti-obesity effects of taurine [6,7]. However, limited data are available regarding the effects of taurine on body weight and obesity in humans [8].

CDO concentration in liver and adipose tissue is regulated by cysteine availability via regulation of CDO degradation [9]. Cysteine dioxygenase might be a co-activator of PPARγ, and is required for adipogenesis via recruitment of PPARγ to the promoters of target genes [10]. PPARγ induces adipogenic differentiation and lipid accumulation by modulating several genes that regulate adipogenesis, lipid uptake and lipid metabolism [11]. The insulin-sensitizing action of PPARγ agonists such as thiazolidinedione results from their ability to regulate the expression of proteins that modulate insulin action and serve as adipose remodeling agents that direct lipids into subcutaneous fat tissue containing small, insulin-responsive adipocytes [11]. Taurine accumulated markedly both intracellularly and in the culture medium of mature adipocytes when medium was supplemented with cysteine [9]. In rats, adipose tissue, like the liver, was shown to be an important site for the regulation of cysteine levels and for hypotaurine/taurine synthesis [9], but little evidence is available in humans. The aim of the present study was to investigate whether the effects of cysteine on adipogenesis [5] are mediated by its catabolic product, taurine.

Material and Methods
The study was conducted at the Center of Excellence for Research in Regenerative Medicine Applications (CERRMA), Alexandria Faculty of Medicine. The experimental protocols were approved by the Medical Ethics Committee (IRB NO: 00012098-FWA- NO: 00018699), Faculty of Medicine, Alexandria University, Egypt.

Human adipose tissue samples
Human white adipose tissue liposapirate (WAT) was obtained during elective abdominal liposuction procedures performed at Alexandria University Hospitals from 3 women who had given written informed consent. Donors were selected to be free of chronic disease as assessed by medical history and routine laboratory tests. Samples were obtained using the Water-Jet Assisted Liposuction (WAL) technique [12], and the sampling conditions during liposuction were optimized to ensure the quality of the cells obtained.

Isolation and culture of adipocyte precursor cells
The protocol for the isolation and culture of adipocyte precursor cells was modified from Bunnell et al [13], the steps are detailed below, and summarized in Figure 1. All cell culture reagents were obtained from Sigma-Aldrich (St. Louis, MO, US). Tissue culture plastics were obtained from Corning Incorporated Life Sciences (Corning, NY, US).

Isolation of adipocyte precursor cells
The liposapirate was washed several times with an equal volume of PBS + antibiotic /antimycotic (100 I.U./mL penicillin, 100 μg/mL streptomycin and 2.50 μg/mL amphotericin) until the adipose layer was yellow in color. For each wash, PBS was added, the bottle was gently swirled to mix, left for separation and infra-natant was aspirated and discarded. The final lipid layer was digested with 0.1% collagenase type IA dissolved in an equal volume of PBS, then filtered. The collagenase/lipid mixture was then placed in a shaking 37 °C water bath for approximately 1 hour, and was gently swirled every 5-10 min to allow better digestion. After digestion, the infranatant containing the SVF was aspirated and an equal volume of complete media was added [DMEM 4.5 g/L glucose with L-glutamine, 10% fetal bovine serum and 1% antibiotics] to inactivate the collagenase, then centrifuged for 10 min at 300xg to collect the pellet of SVF. All SVF pellets were collected into one centrifuge tube, passed over 100 μm cell strainer, then centrifuged for 5 min at 300xg. Lysis of RBCs was done to yield a clear SVF pellet.

Cell culture and monitoring
After isolation, cells were then counted, and seeded into a 12- well plate (seeding density: 30000 cell/cm2) in complete media in 37c 5% CO2 incubator. The cells were monitored daily using an inverted phase contrast microscope, and the media was changed every 2 days. When the cells reached 75-80% confluence, the adipocyte differentiation protocol was applied. The cells were then daily monitored until the 8th day of differentiation. Samples for PCR were collected on days 0 and 4 of differentiation, while Oil Red O staining was performed on day 8 (end of the differentiation protocol).

Differentiation of adipocyte precursor cells at different cysteine concentrations
Methionine- and cysteine-deplete DMEM (Sigma-Aldrich #D0422) supplemented with 30 μM-methionine (Sigma-Aldrich #M5308) and variable concentrations (10-50 μM) of L-cystine (Sigma-Aldrich #C7602) from individual stock solutions of L-methionine (20 mM) dissolved in H2O and L-cystine (10 mM) dissolved in 0.2 M HCl, were used. The concentration of cysteine and methionine (30 μM) was selected based on previous studies in 3T3-L1 adipocytes [5].

To induce differentiation, cells were treated (on day 0) with an induction medium [13] (containing 1 μM dexamethasone, 58 μg/mL insulin, 0.5 mM 3-isobutyl-1-methylxanthine, and 200 μM indomethacin) and media was replaced with fresh induction media on day 2. On day 4, the cells were treated with insulin medium (containing 10 μg/mL insulin), and the medium
was changed every 2 days till day 8, when clusters of mature adipocytes filled with lipid droplets were visible.

Effect of taurine supplementation on lipid accumulation and adipogenic gene expression.

To test the hypothesis that the CDO product, taurine, is the mediator of cystine effect on adipogenesis, human SVF was cultured under low cystine concentrations (15 μM) with or without 30 μM taurine supplementation. Then, Oil Red O staining and gene expression analysis of CDO1 and PPARG1 mRNA were assessed.

Reverse transcription-quantitative polymerase chain reaction (RTqPCR)

Isolation and reverse transcription of RNA

On day 0 and day 4 of differentiation, cells were washed twice with ice-cold PBS, lysed by 500 μL Qiazole and frozen at −80 °C until RNA isolation. Total RNA was isolated using a spin protocol (Qiagen RNeasy Mini Kit #74104). RNA concentrations and quality (260/280 ratio) were determined on a Nanodrop Spectrophotometer and stored at −80 °C. Total RNA (12.5–25 ng/μL) was reverse transcribed using a high-capacity cDNA reverse transcription kit (Life Technologies #4374966) on an Applied Biosystems GeneAmp PCR System thermal cycler with the following settings: 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 s, and 4 °C on hold. cDNA was stored at -20 until qPCR experiments.

qPCR and data quantification

Gene-specific regions were amplified from cDNA (5–10 ng/μL) with primers (100 nM each; Biosearch Technologies (Novato, CA, US)) and Maxima SYBR Green/ROX kit (Thermo Scientific #K0221) on an Applied Biosystems™ StepOne™ Real-Time PCR System with the following settings: 25 μL reaction, 95°C for 10 min, followed by 40 cycles; 95°C for 15s, 60°C for 30s and 72°C for 30 s. Gene expression analysis was performed using the relative quantification (ΔΔCt) method. Results are presented as fold change relative to β-actin (2−ΔΔCt). The primer sequences are listed in Table (1).

Oil Red O triglyceride staining

To assess lipid accumulation, Oil Red O staining was performed on differentiated mature adipocytes on the 8th day of differentiation as described [15]. Images were taken with an inverted camera-equipped microscope (Olympus CKX41) at 200x magnification and analyzed for mean lipid droplet size and percentage of lipid area using Fiji image analysis software (NIH, Bethesda, USA).

Table 1. Primer sequences used in quantitative real-time PCR (qRT-PCR).

<table>
<thead>
<tr>
<th>GENE</th>
<th>Primer sequences</th>
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<tbody>
<tr>
<td>PPARG1</td>
<td>Fwd: 5’-CGAGACACCGCGAGAGG-3’&lt;br&gt;Rev: 5’-TGTGGTTAGTGTTGGCTTCTT-3’</td>
</tr>
<tr>
<td>CDO1</td>
<td>Fwd: 5’-TCTCTGTGCTGCGTAAGGAC-3’&lt;br&gt;Rev: 5’-GCCAGGCAAATAATGTCTCC-3’</td>
</tr>
<tr>
<td>β-ACTIN</td>
<td>Fwd: 5’-TGCCGACCCAGCAATTAGTCTCC-3’&lt;br&gt;Rev: 5’-CTAAGCTATAGTCCGCCCTAGAGCA-3’</td>
</tr>
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Statistical analysis

Data are presented as mean ± SEM and are compared across groups using Student’s independent t-test. P<0.05 was considered statistically significant. GraphPad Prism (version 8.3.1. for Windows) was used for data analysis.

Results

Morphologic appearance of differentiated mature adipocytes in culture on days 3-8 of differentiation

Morphologic changes were closely monitored during differentiation. Starting from the 3rd day of differentiation, small lipids droplets started to appear. On the 8th day of differentiation, the cells rounded up to a more spherical shape from a more elongated fibroblast shape, and clusters of mature adipocytes filled with lipid droplets were clearly visible. Adipocyte number and lipid droplet size increased in differentiated mature adipocytes cultured at ascending cystine concentrations.

Effect of taurine supplementation on lipid accumulation

In view of previous findings that taurine is markedly accumulated both intracellularly and in the culture medium of mature adipocytes, but not preadipocytes in response to cysteine [9], we sought to test the hypothesis that the CDO product, taurine, is the mediator of cystine effect on adipogenesis [5]. Morphologically, Oil Red O staining showed no difference in cells differentiated at low cystine with or without taurine supplementation (Figure 2. A). Quantification of the percentage lipid area stained revealed no significant difference in response to taurine supplementation (P= 0.97), (Figure 2.B). There was also no significant difference in the size of lipid droplets in cells differentiated in taurine-supplemented media versus non-supplemented media (P =0.251) (Figure 2.C).

Effect of taurine supplementation on adipogenic gene expression

Gene expression analysis also revealed that 30 μM taurine supplementation in the culture medium showed no significant increase in expression of PPARG (p=0.918) and CDO mRNA (p=0.086) compared to low cystine (Figure 3).
Discussion

Evidence from epidemiological studies, animal models and murine adipocytes suggest that cyst(e)ine availability is related to adiposity and enhanced adipogenic differentiation [3,5]. However, the mechanisms of cystine effect on adipogenesis need further investigations. Taurine, a catabolic product of cysteine is thought to play a role in adipogenesis. The aim of our study was to investigate whether the effect of cystine on adipogenesis [5] is mediated through its catabolic product, taurine.

Extracellular cystine was previously shown to enhance adipogenic differentiation and PPARg expression in 3T3L1 cells in a dose-dependent manner [5]. In the current study, we started by testing similar cystine concentrations (10–50 μM)
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on the differentiation of human preadipocytes [5]. To induce differentiation of preadipocytes, our differentiation cocktail [13] included insulin, dexamethasone, IBMX. Indomethacin was used as PPARg agonist instead of rosiglitazone, since indomethacin has been shown to enhance adipogenic differentiation via multiple prostaglandin-dependent and independent mechanisms, in addition to PPARg induction [15,16]. Indomethacin also increases PPARg2 protein to levels that are higher than those observed with rosiglitazone, suggesting an effect of indomethacin on PPARg2 through post-transcriptional mechanisms [16].

Ueki I et al studied the pathways for taurine synthesis in 3T3-L1 cells during their adipogenic conversion. It was noted that CDO mRNA levels increased during adipogenic differentiation in response to cysteine or cysteamine treatment in adipocytes and were accompanied by increased taurine production [2], which suggests a role of taurine in adipogenesis. In the present study, primary human SVF including preadipocytes were cultured under limiting cystine concentrations with or without 30 μM taurine supplementation. The concentration of taurine was chosen to be similar to that reported in human plasma [17]. Our data showed no significant effect of taurine supplementation on lipid accumulation, as assessed by quantification of total area stained and mean lipid droplet size. Taurine supplementation in the culture medium also had no significant effect on the mRNA expression of PPARg during adipogenesis. In support of our findings, Hou et al showed that, although the taurine transporter played an important role in the differentiation of human adipose-derived stem cells into adipocytes, alongside its substrates, hypotaurine and β-alanine, taurine failed to enhance adipogenesis in the same model [18].

The effect of taurine supplementation on CDO expression deserves further investigations. In our study, a non-significant trend for increased CDO gene expression was observed following taurine supplementation. The reason for this is unclear. Studies showed that adding taurine to cells grown in a taurine-free medium has little direct effect on CDO gene transcript levels. In contrast, taurine supplementation reduces transcript levels of the taurine transporter, TauT [19]. CDO is also known to be regulated post-transcriptionally in response to changes in intracellular cysteine concentration via changes in the rate of CDO ubiquitination and degradation [20], the possibility of a similar post-transcriptional regulation by taurine warrants investigations.

The association between taurine and adiposity in animal and human studies is complex. While numerous studies have noted an anti-obesity effects of taurine in rodents, the evidence in humans is less clear. An early study on genetically obese mice, showed that twenty-week supplementation of taurine in genetically obese/hyperglycemic KK mice reduced body weight gain and abdominal fat compared with control KK mice [21]. Moreover, Tsunoyama-Kasaoka et al demonstrated that dietary taurine supplementation prevented high-fat diet-induced obesity and increased resting energy expenditure in mice [7]. Taurine was also shown to prevent obesity and to improve glucose tolerance in weaned mice fed a high-fat diet for 8 weeks [22]. Additionally, taurine supplementation for 14 weeks significantly reduced body weight gain and weight of the white adipose tissues in mice fed a high-fat diet, and reduced the infiltration of macrophages and the production of inflammatory cytokines [6]. Taken together, these observations suggest a possible role of taurine in the prevention of diet-induced obesity. Human studies, however, have failed to collectively document a clear anti-obesity effect of taurine supplementation. An early cross-sectional study in 60 populations across 25 countries worldwide found that 24-hour urinary taurine excretion, which is considered a marker for dietary taurine intake, was inversely associated with BMI and other markers of cardiometabolic risk [23]. However, this finding may be explained by confounding, since the intake of at least one taurine-rich food, namely fish, is itself inversely associated with BMI [24]. Indeed, a meta-analysis of 12 randomized controlled trials of taurine supplementation for durations ranging from 15 days to 6 months concluded that taurine has no significant effect on body mass index (BMI) [25], at doses ranging from 0.5 to 6 g/d. This conclusion is in line with our observation that taurine did not significantly influence human adipogenesis in the present study.

The main strength of the study is that primary preadipocytes were derived from volunteers of similar age, sex, BMI and ethnicity. The tested extracellular taurine concentration was physiologic, and in line with reported taurine levels in plasma [17]. The study was designed primarily for proof of concept and more work is needed for elucidation of downstream mediators. Furthermore, while taurine had no significant effect on adipogenesis in our in vitro model, it cannot be determined conclusively that the same occurs in vivo, where metabolic, endocrine, and paracrine stimuli could modify its effects on preadipocytes.

In conclusion, taurine supplementation at concentrations similar to those observed in human plasma did not stimulate adipogenic differentiation in preadipocytes cultured at limiting cysteine concentrations. This suggests that previously reported effects of extracellular cystine on adipogenesis in 3T3-L1 cells [5] may be independent of its catabolic product, taurine. The mechanistic effects of different sulfur containing compounds on human adipogenesis deserve further investigations.

Scientific Responsibility Statement
The authors declare that they are responsible for the article’s scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and human rights statement
All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.

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Conflict of interest
None of the authors received any type of financial support that could be considered potential conflict of interest regarding the manuscript or its submission.

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